

# **Evaluating Poloxamers as Agents for Acceleration of Skeletal Muscle Membrane Repair**

Undergraduate Research Thesis

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## Abstract

Sarcolemmal membrane fragility is a major contributor to the pathology of various muscular dystrophies, including Duchenne Muscular Dystrophy (DMD). DMD is a fatal X-linked genetic disorder resulting from a loss of the dystrophin protein. This causes muscle degeneration and weakness, in both skeletal and cardiac tissue. Muscle fibers with more fragile membranes experience a higher degree of damage and are more prone to necrotic cell death. Increasing the membrane repair capacity of these fibers can serve as a potential therapeutic by effectively restoring the barrier function of the damaged membrane and prevent muscle fiber death and muscle loss. One strategy to increase the membrane repair capacity is to expose the muscle fibers to Poloxamer 188 (P188), a polymer with affinity to exposed hydrophobic lipid chains that can reseal membrane wounds. P188 is part of a family of poloxamers, all of which contain a hydrophobic region of polyoxypropylene flanked by two hydrophilic chains of polyoxyethylene. These regions vary in length between different poloxamers. Although P188 has shown promising results in membrane repair, other poloxamers in this family have not been tested for their membrane sealing capacity. It is hypothesized that other poloxamers in the P188 family such as F38, P84, and P407 will reseal membrane wounds as or more effectively than P188.

To investigate this hypothesis, we used both a rotation damage assay and a laser injury assay to examine the repair efficiency of poloxamers on *in vitro* human embryonic kidney (HEK293) cells and *ex vivo* muscle fibers isolated from *mdx* mice. Studying the effect of these compounds on muscle fibers with compromised membrane integrity allowed for the evaluation of these poloxamers as potential therapeutic agents for muscular dystrophy.

The conclusion of these assays indicates that there are poloxamers other than P188 that can improve the repair capacity in both HEK293 cells and dystrophic *mdx* muscle fibers.

## **Introduction**

Muscular dystrophies come in a variety of forms, all characterized by muscle weakness and degeneration. One of the most common and most severe forms of muscular dystrophy is Duchenne Muscular Dystrophy (DMD) (1). DMD is a genetic disorder affecting one in every 3,500 to 5,000 males (2). Affected individuals display severe muscle weakness in early development, indicated by delays in sitting up and walking. In their early teens, patients often experience heart and lung problems, resulting from muscle weakness in the heart, diaphragm, and other muscles surrounding the lungs. DMD is a fatal disease, but with the advancement of respirators the life span of affected individuals can extend into their 30s and 40s (3).

Duchenne Muscular Dystrophy is an X-linked, recessive disorder, resulting from mutations in the *DMD* gene. The *DMD* gene encodes dystrophin, a protein that forms a complex to help stabilize the cytoskeleton and form connections with the extracellular matrix (2,4). Large disruptions of this protein are thought to lead to nonsense-mediated decay, and complete loss of the dystrophin protein (5). Loss of this protein causes increased sarcolemmal membrane fragility, leaving cells susceptible to tears caused by muscle contraction. Muscle fibers are typically able to reseal membrane disruptions through an inherent  $\text{Ca}^{2+}$ -mediated repair system, in which vesicles fuse to the injury site to form a patch that restores the barrier function of the sarcolemma (6–8). In DMD, the membrane repair response fails to keep up with the increase in mechanical damage, and

this damage ultimately results in uncontrolled ion influx and cell death (9). A potential therapeutic technique is to improve the membrane repair response, thus allowing cells to more effectively recover from damage. One way to enhance membrane repair is through exposure to Poloxamer 188 (P188).

P188 is a member of a family of poloxamers, all of which have been approved by the FDA. Poloxamers are synthetic copolymers, made up of two hydrophilic polyoxyethylene chains flanking a hydrophobic polyoxypropylene chain (10) (Fig 1A). These chains vary in size between different poloxamers. These differences can be visualized through the use of a poloxamer grid, where poloxamers are categorized based on the weight of their polyoxypropylene (POP) chain on the y-axis, and the % weight of their polyoxyethylene (POE) chains on the x-axis (Fig 1B). Poloxamers have many everyday uses, and can be found in products such as moisturizers, makeups, hair conditioners, and mouthwashes (11). Certain poloxamers have also been studied in medical applications. Specifically, P407 has suggested applications in drug delivery and as a scaffold for tissue engineering (12). P407 has also been used in combination with P181 to increase expression of plasmid DNA when used in delivery to skeletal muscle cells (13).

P188 is the most common poloxamer used in biomedical applications. It has been extensively studied because of its ability to improve cell membrane repair. This characteristic has been observed for more than 20 years, since P188 was first recognized for its ability to reseal tissue after electrical injury (14). It has since been tested in the repair of multiple cell types following various types of injuries (15,16). Specifically, P188 has been shown to improve repair of alveolus cells in injured lungs and muscle cells following irradiation (17). It also has been noted for its ability to improve survival of

neurons following both mechanical and ischemia/reperfusion injury (18,19). More recent studies indicate that administration of P188 *in vivo* can alleviate symptoms in models of Duchenne Muscular Dystrophy. These findings suggest that P188 has a protective effect on skeletal muscle when delivered subcutaneously, and on cardiac muscle when delivered intravenously (9,20–22). While the mechanism of this improved repair is unknown, it is hypothesized that P188 functions by directly incorporating into the membrane (23).

Despite their structural similarities to P188, other poloxamers remain largely untested in terms of membrane repair effects. This project examined a subset of these poloxamers to determine their effects on membrane repair. I hypothesize that multiple poloxamers in the P188 family will improve membrane resealing in multiple cell types as effectively as P188. Poloxamers P181, P182, P234, P124, P407, P338, P188, and P108 were all selected for analysis (Fig 1C). These poloxamers were chosen to best represent the wide range of poloxamers that are produced. This allows us to compare changes in membrane resealing capacity between large and small poloxamers, and assess the effect of different POP and POE weight ratios.

## **Materials and Methods**

P181: Sipectrum-P1162  
P124: Spectrum-P1168  
P182: Spectrum-P1818  
P234: Aldrich-713538-1KG  
P108: BASF-51188659  
P188: Sigma-15759-1KG  
P407: Sigma-16758-250G  
P338: BASF-50967755

Cell Culturing: Human embryonic kidney cells (HEK293) were purchased from American Type Culture Collection (ATCC). The cells were cultured at 37°C and 5% CO<sub>2</sub> in complete medium composed of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum, (FBS) and 1% penicillin–streptomycin (P–S). Cells were split onto new plates at 90% confluency and used for examination until passage 25.

Rotation Damage Assay:  $1 \times 10^5$  HEK293 cells in 500µL of complete DMEM were added to 2mL microcentrifuge tubes and incubated for 18 hours. This allowed cells to adhere to the bottom of the tube. Cells were rinsed with 500µL PBS before adding the necessary reagents to each group. Each group contained three tubes of HEK293 cells. 200µL of 1% Triton in PBS was added to one group, in order to lyse the cells. This was the maximum control. Tubes used for the 15 rotation and no rotation controls received 200µL Tyrode's solution with 2mM Ca<sup>2+</sup>. The remainder of the tubes served as experimental groups for various poloxamers. In these tubes, 200µL Tyrode's solution with 2mM Ca<sup>2+</sup> was added with the addition of 100µM poloxamer. 30µL glass beads ( $\geq 106\mu\text{m}$ ) were added to each tube except for the no rotation control, and tubes with beads were rotated 360° 15 times, at a rate of ~4 seconds per revolution. As the tubes rotated, the beads impacted the cells at the bottom of the tube, resulting in injury to the cell membranes. After rotating, 15µL supernatant was removed from each tube, and added to a 96 well plate in duplicate. The relative lactate dehydrogenase (LDH) concentrations were measured using colorimetry of the reduction reaction between NAD<sup>+</sup> to NADH in the presence of L-lactate at 490nm. Absorbance values were normalized to the values obtained from supernatant of cells lysed with 1% Triton.

Laser Injury Assay: Extensor digitorum longus (EDL) muscles were dissected from *mdx* mice. Muscles were adhered to a 35mm glass bottom plate, and submerged in 500 $\mu$ L Tyrode's solution containing 2mM  $\text{Ca}^{2+}$ , 100 $\mu$ M poloxamer, and 2.5 $\mu$ M FM4-64 dye (ThermoFisher-T13320). Muscle fibers were imaged individually using an Olympus Multiphoton FV1000 microscope. A UV laser was used to vaporize an area of the cell membrane, with a size of about 0.9 $\mu$ m x 0.9 $\mu$ m. Images were taken every 1.5 seconds for a period of 60 seconds, with the first three frames captured prior to injury. Data was analyzed using ImageJ software by measuring fluorescence intensity at the injury site and calculating the change in fluorescence intensity over time. The fluorescence intensity is normalized to the background intensity in each image ( $\Delta F/F_0$ ).

Membrane resealing in HEK293 cells was also assessed through laser injury. Cells were cultured overnight to about 80% confluence, and washed with 1mL PBS. 2mL of Tyrode's solution containing 2mM  $\text{Ca}^{2+}$ , 100 $\mu$ M poloxamer, and 2.5 $\mu$ M FM4-64 dye was added to the cell plate, and the cells were damaged, imaged, and analyzed similarly to EDL muscle fibers. Images of HEK293 cells were captured every 1.5 seconds for a period of only 45 seconds.

## **Results**

A novel rotation damage assay was used to examine the membrane resealing effects of various poloxamers in the P188 family. In this assay, human embryonic kidney (HEK293) cells are damaged through rotation in the presence of small glass beads. These beads impact the cells, causing injury to the cell membranes. This damage leads to release of LDH into the cell supernatant. LDH is typically confined inside the cell, only released after

membrane damage or cell death. Since injury to cells is consistent in each tube, changes in the amount of LDH in the supernatant indicate changes in membrane repair capacity. Membrane repair is a  $\text{Ca}^{2+}$  dependent process, therefore cells were also damaged in Tyrode's solution containing 0mM  $\text{Ca}^{2+}$  to confirm the validity of using LDH release to measure membrane repair capacity. All groups were normalized to the supernatant from cells lysed with 1% triton, which represented maximum LDH release. To investigate the impact of poloxamers on membrane repair, cells were damaged in the presence of 100 $\mu\text{M}$  poloxamer. A diagram and results of the rotation damage assay are shown in figure 2.

Based on the results of the rotation damage assay, P188 significantly reduced LDH release from injured cells, indicating improved membrane repair capacity in HEK293 cells when compared to a no poloxamer control. Poloxamers P182, P108, P234, and P124 also improve membrane repair when compared to cells damaged in the absence of poloxamer. However, none of these poloxamers improve membrane repair significantly more than P188. Poloxamers P181, P338, and P407 led to no significant improvement in membrane repair capacity in HEK293 cells when compared to the 15 rotation control.

A laser injury assay was used to examine the effects of the same group of poloxamers when applied to *mdx* muscle fibers. For this assay, EDL muscles were dissection from *mdx* mice and submerged in Tyrode's solution containing 2mM  $\text{Ca}^{2+}$  and 2.5 $\mu\text{M}$  FM4-64 Dye. FM4-64 is a lipophilic dye that fluoresces when it comes in contact with the inner leaflet of the cell membrane. Individual muscle fibers are damaged with a multiphoton laser, allowing FM4-64 to come in contact with the inner leaflet and fluoresce. The fiber is imaged before, during, and after injury, and the change in fluorescence is measured over time. This fluorescence is normalized to the background fluorescence in each image.



Muscles were also injured in Tyrode's solution with 0mM  $\text{Ca}^{2+}$  to confirm the assay's measurement of membrane resealing. 100 $\mu\text{M}$  poloxamer was added to the Tyrode's solution to measure the effects of poloxamers on membrane resealing capacity in muscle fibers. Results and representative images from laser injury in muscle fibers are shown in Figures 3 and 4. Curves depicting  $\Delta F/F_0$  over time for each poloxamer are shown in figures 3A and 4A. For each curve, area under the curve (AUC) was calculated. The AUC for each curve is shown in figures 3B and 4B.

Figure 3 shows laser injury results of poloxamers with less than 50% of their weight consisting from the POE segments (P181, P182, P234, P124). This set of poloxamers possess mostly hydrophobic properties due to the majority of their weight is comprised of POP chain. Results of the laser injury show that muscle fibers injured in the presence of 100 $\mu\text{M}$  of each respective poloxamer had significantly less FM4-64 dye fluorescence after injury when compared to muscle fibers injured in the absence of any poloxamer. This suggests that all of these poloxamers improve membrane repair capacity in *mdx* muscle fibers.

Figure 4 shows laser injury results of poloxamers with more than 50% of their weight made up of POE (P188, P407, P338, P108). These poloxamers are more hydrophilic, since most of their weight is composed by the POE chains on either end of the polymer. Results of the laser injury assay reveal that out of this group of poloxamers, only P188 and P407 lead to a significant improvement in membrane repair capacity of *mdx* muscle fibers. Analysis by ANOVA indicates that none of the poloxamers investigated as part of this project improved membrane repair significantly better than P188.

Interestingly, some poloxamers affected membrane repair differently in the two injury assays used as part of this project. For example, P108 significantly improved membrane repair capacity in HEK293 cells when investigated using the rotation damage assay, but had no effect on membrane repair when applied to muscle fibers in the laser injury assay. Conversely, P407 and P181 had no effect when examined in the rotation damage assay with HEK293 cells, but muscle fibers injured in the presence of each of these poloxamers showed significant improvement in membrane repair. These differences could be explained in two ways. First, the specificity of these poloxamers is due to the type of membrane damage induced (assay dependent) or they are selective to different cell-types.

To distinguish if the poloxamers function in a cell type or wound type manner, the repair capacity of HEK293 exposed to these poloxamers were analyzed through the laser injury assay. This is done using the same technique described above, however, using HEK293 cells rather than muscle fibers. The results of these experiments are displayed in Figure 5. P108 was chosen for examination because it is one of the poloxamers that had different effects in the laser injury and rotation damage assays. The results of this laser injury assay indicate that exposure to 100 $\mu$ M P108 leads to a significant decrease in FM4-64 fluorescence after injury to HEK293 cells. Since exposure to P108 improves membrane repair in HEK293 cells in both the rotation damage and the laser injury assays, these results suggest that P108 improves membrane repair capacity in a cell-type specific manner.

## Discussion

The primary goal of this project was to identify other poloxamers in the P188 family with the ability to improve membrane resealing capacity. If other poloxamers have effects similar to those of P188, these poloxamers may have potential as therapeutics for diseases such as Duchenne Muscular Dystrophy.

Results suggest that a number of poloxamers improve membrane repair in both the rotation damage assay, performed with HEK293 cells, and the laser injury assay, performed with muscle fibers. These are poloxamers P182, P124, P234, and P188. Poloxamers P182, P124, and P234 all have weights consisting of less than 50% POE. This makes these poloxamers more hydrophobic and giving them a strong affinity for the lipids that are exposed after damage occurs to a cell membrane. P188 has more than 50% POE by weight, making it a more hydrophilic molecule. However, the number of POP repeats in P188 is within the same range of P182, P124, and P234. Each of these poloxamers has between 20 and 41 POP repeats. Each of these poloxamers also has between 8 and 80 POE repeats. P182 has the smallest number of POE repeats with 8, and P188 has the most repeats with 80 (24).

Poloxamer P108 was the only polymer to improve membrane resealing in HEK293 cells, but not in muscle fibers. As previously mentioned, the results summarized in figure 5 suggest that this difference is a result of P108 having cell-type specific effects. P108 consists of 43 POE repeats, and only 15 POP repeats, making this poloxamer more hydrophilic. While the number of POE repeats falls in the same range as the poloxamers that improved membrane repair in both cell types, P108 has the smallest number of POP repeats out of all poloxamers tested. One explanation could be that the POP segment is

too small and has a less robust affinity to the hydrophobic lipid chains in the plasma membrane. Because the membrane of HEK293 cells have a large surface area directly exposed to the solution, poloxamers with less attraction to membrane wounds can still bind and restore the cell's barrier function. However, in *mdx* muscle fibers, which exhibit compromised membrane repair and stability, it may be more important to have a longer hydrophobic chain that can seal a membrane with more exposed lipids after injury.

Poloxamers P181 and P407 each improved membrane resealing in *mdx* muscle fibers, but not in HEK293 cells. P181 has the smallest molecular weight of all poloxamers tested, and consists of 30 POP repeats, and only 3 POE repeats. This composition makes P181 a mostly hydrophobic polymer. With such short POE chains, it is possible that this poloxamer has a very low affinity for the hydrophilic region of an injured cell membrane, which may affect its ability to improve resealing capacity of HEK293 cells. P407 is one of the largest poloxamers tested, consisting of 101 POE repeats and 56 POP repeats. This composition makes this poloxamer more hydrophilic. The large number of POP repeats may make the hydrophobic region too large to incorporate into a normal injured cell membrane. However, this large POP region may make this more effective in resealing injuries that occur to cells with a membrane repair defect or less membrane integrity. This would explain why P407 was effective in improving resealing capacity of *mdx* muscle fibers, but not in HEK293 cells.

Of all poloxamers tested, only P338 had no effect on membrane repair capacity in either cell type. P338 has the largest molecular weight of all poloxamers tested, consisting of 132 POE repeats on either side of the polymer, and only 51 POP repeats in the center.

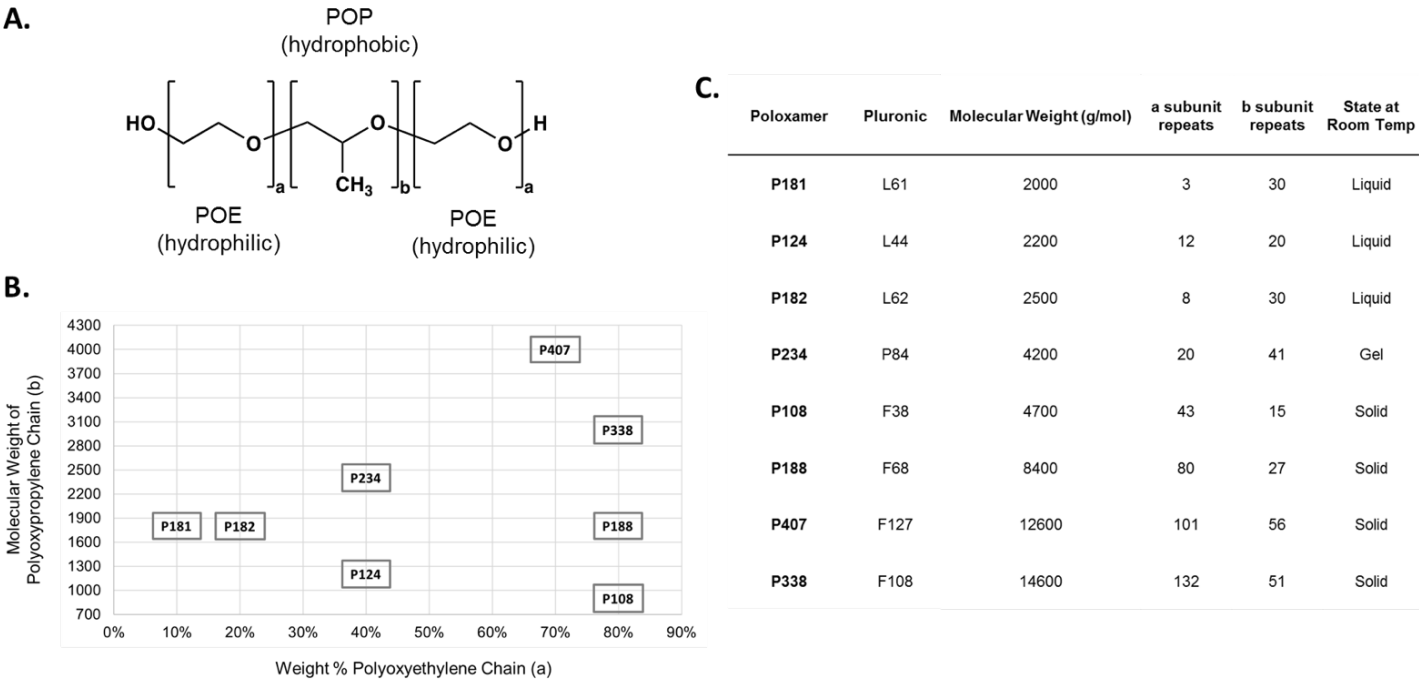
This is the largest number of POE repeats of all poloxamers tested, and it's possible that these large hydrophilic chains hinder the poloxamer's ability to reseal membrane wounds.

While this project provides a preliminary investigation into the membrane resealing effects of multiple poloxamers, more research is required to understand these poloxamers' potential as therapeutics for diseases such as muscular dystrophy. Past investigations into the effects of P188 have used a number of experiments that could be useful when looking at additional poloxamers. Specifically, studies have used contraction-induced injury to determine effects of P188 on skeletal muscle (25). *In vivo*, the effects of P188 have been investigated with electroporation injury in mice. Results of this study suggest that intravenous injection of P188 can facilitate recovery of muscles after injury *in vivo* (15). Similar experiments could be used to investigate the effects of other poloxamers *in vivo*, specifically in *mdx* mice.

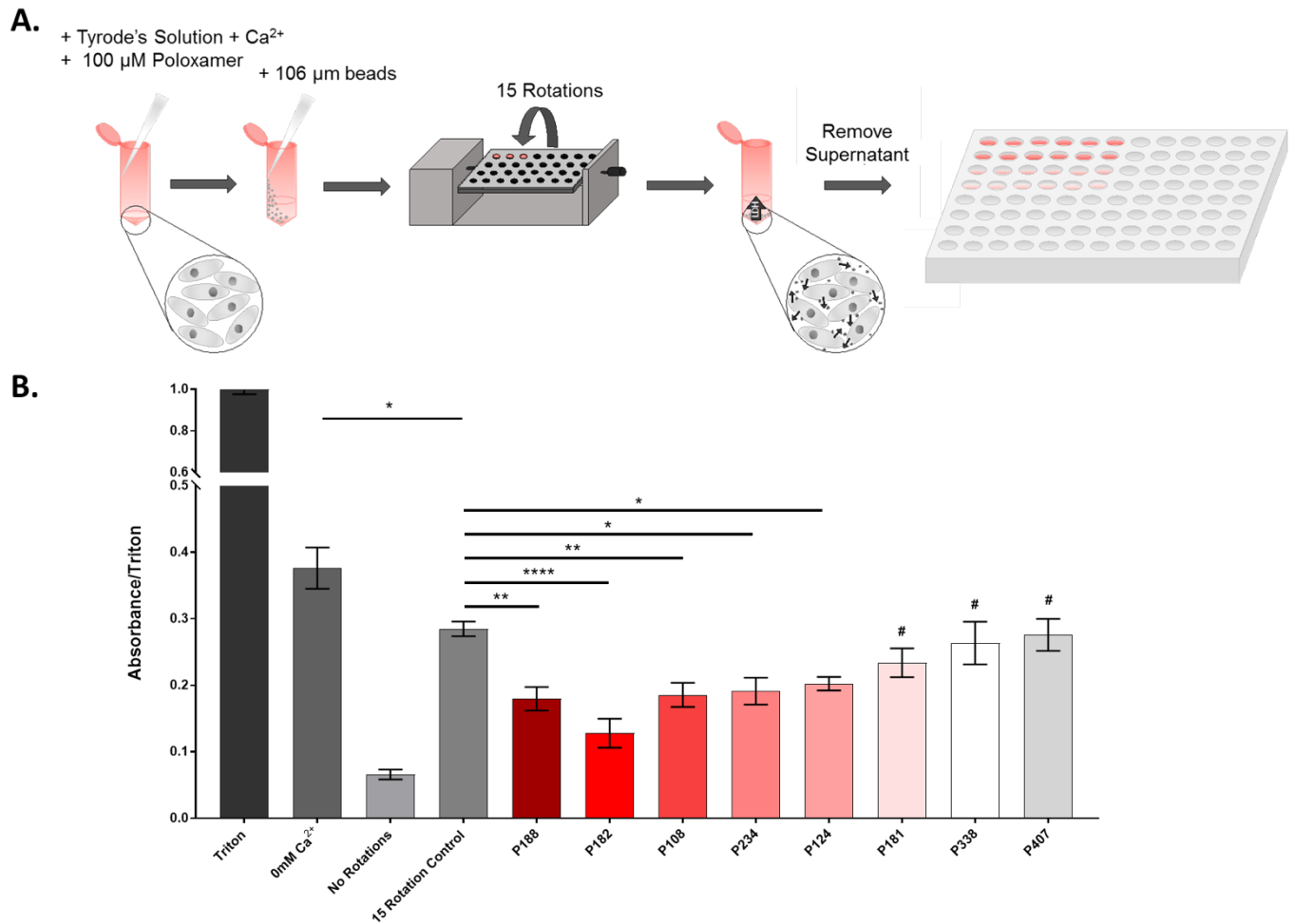
Additionally, new poloxamers could be synthesized with a similar general structure, but different variations in the number of POP and POE repeats. It would be particularly useful to obtain poloxamers with more varied ratios of POP to POE. For example, no poloxamers were examined with a POE weight % of less than 30% and a POP molecular weight above 2500 g/mol (fig 1A). There were also no poloxamers tested with either 50% or 60% POE by weight. Synthesizing and testing more poloxamers in these regions could help further understand the mechanisms behind the cell-type specific membrane repair observed in these experiments.

## Conclusion

With many groups investigating the membrane resealing effects of P188, it is important to understand the effects of other poloxamers in the P188 family. The structural similarities between P188 and other poloxamers in the P188 family make these polymers good potential candidates for improving resealing capacity in multiple cell types. Our results suggest that poloxamers with intermediate POP and POE lengths are most effective at improving membrane repair across multiple cell types. Poloxamers with either longer or shorter POP and POE chains seemed to improve membrane repair in a more cell-type dependent manner. The largest poloxamer tested, P338, was the only poloxamer to show no signs of improving membrane repair capacity in either HEK293 cells or *mdx* muscle fibers. Overall, results suggest that there are multiple poloxamers in the P188 family that have the ability to improve membrane repair capacity. To determine the potential of these poloxamers as therapeutics, future investigations should continue to evaluate these polymers, and others, in *in vivo* models of muscular dystrophy.

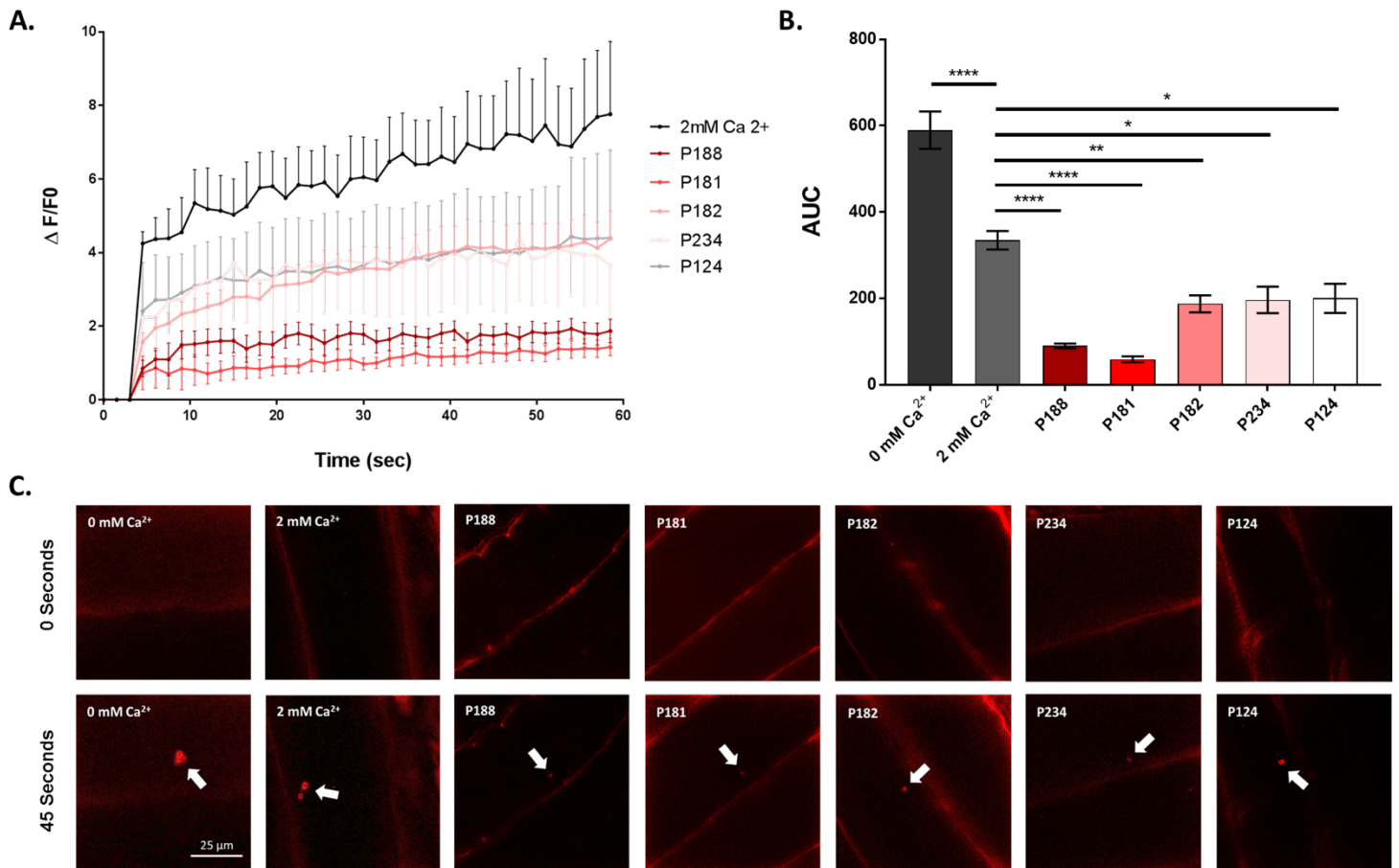


**Figure 1. A.** General structure of a poloxamer. This image shows the molecular makeup of a poloxamer, with two chains of polyoxyethylene (POE) flanking a chain of polyoxypropylene (POP) **B.** Poloxamer Grid. This is a visual representation of how different poloxamers compare to each other. Poloxamers are characterized by the weight % of the POE chain and the molecular weight of the POP chain. **C.** Structure of Poloxamers Screened. This table gives an overview of the size and physical properties of each poloxamer, including molecular weight and the size of both the POE (a) repeats and the POP (b) repeats.

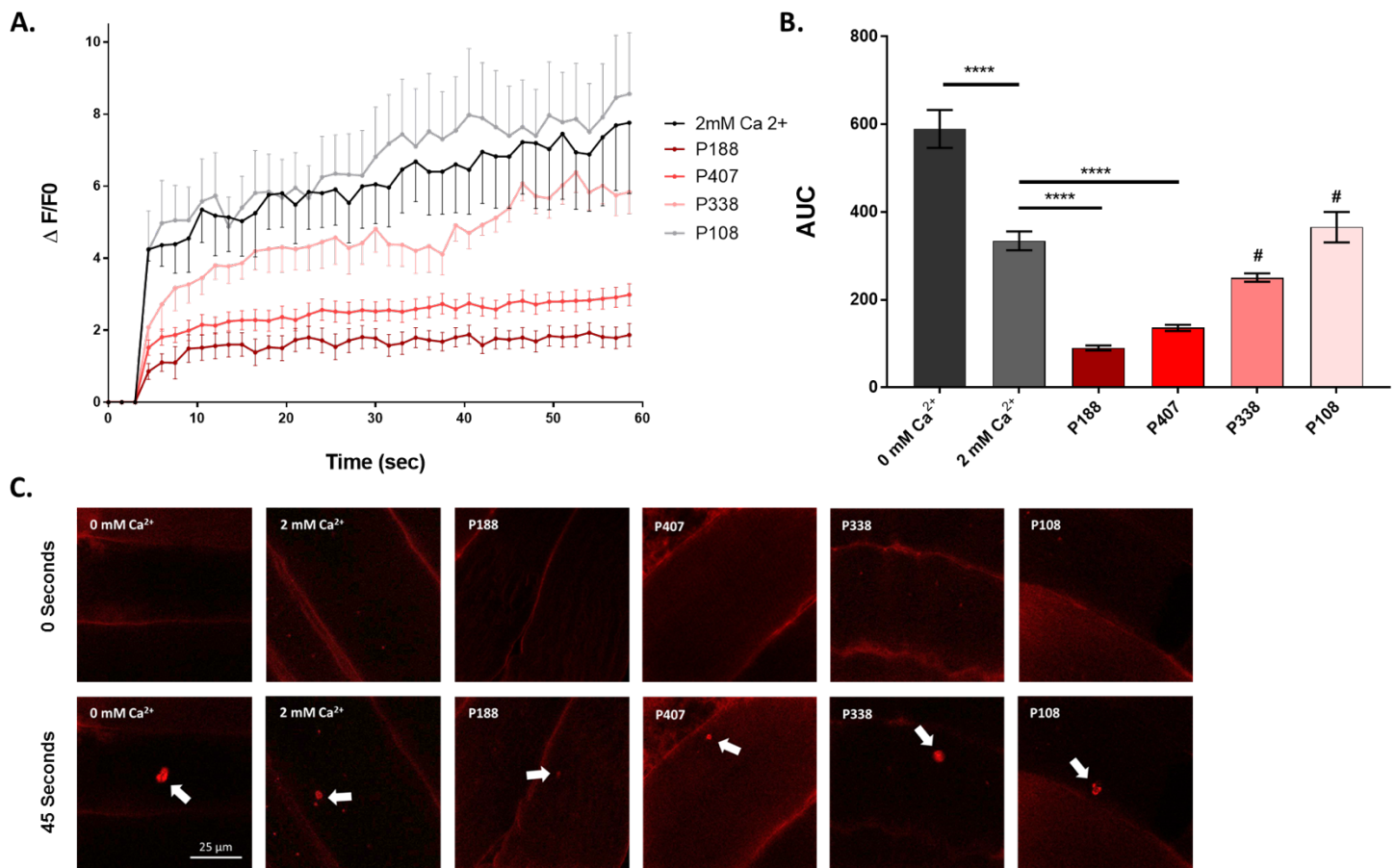


**Figure 2. A.** Diagram depicting rotation damage assay. HEK293 cells are cultured overnight in 2mL microtubes to allow attachment to the bottom of the tube. The cells are washed with PBS and 20  $\mu\text{L}$  of small glass beads ( $\leq 106\mu\text{m}$ ) are added to each sample. Tubes are rotated 15 times at a rate of 1 revolution per 4 seconds to damage the samples. 15  $\mu\text{L}$  of the supernatant is collected and the amount of leaked LDH released is measured. Each of the samples are normalized by the total LDH release produced by triton detergent. **B.** Results of the rotation damage assay, showing the absorbance at a wavelength of 490 after an LDH test was performed on the supernatant as described in figure 2A. Graph shows mean with SEM. Analysis by one way ANOVA comparing to the 15 rotation control. \* $P \leq 0.05$  by ANOVA, \*\* $P \leq 0.003$  by ANOVA, \*\*\*\* $P \leq 0.0001$  by ANOVA, # $P > 0.05$  by ANOVA,  $n=12-42$

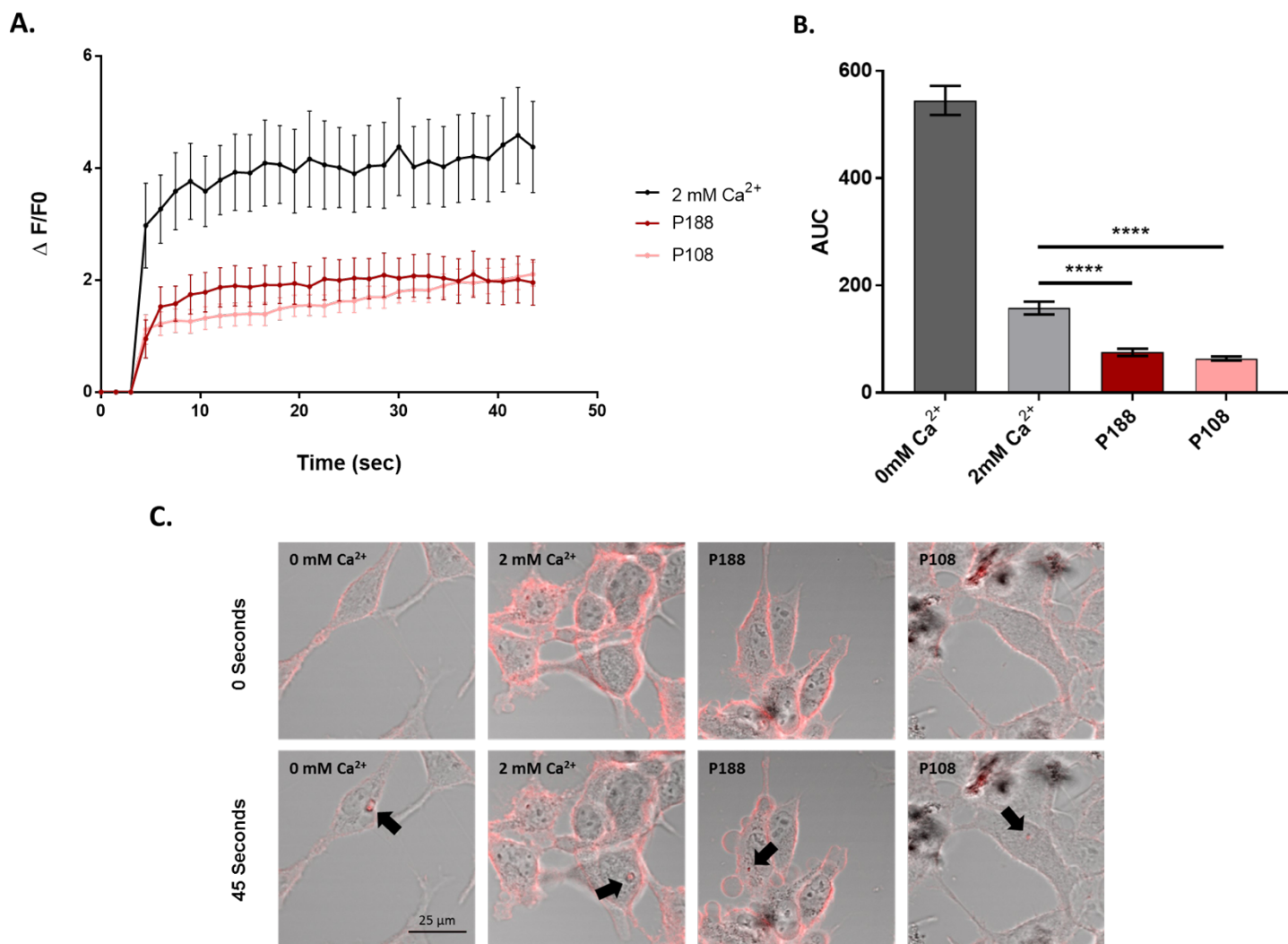




**Figure 3. A.** Results of laser injury assay performed in extensor digitorum longus (EDL) muscles of *mdx* mice, when exposed to poloxamers with less than 50% POE by weight. All curves represent fluorescence after injury while muscle was in 1X Tyrode's solution with 2mM  $\text{Ca}^{2+}$  and 100 $\mu\text{M}$  poloxamer. Graph depicts change in fluorescence of a lipophilic dye, (FM4-64) over time at the injury site with SEM. **B.** Area Under the Curve (AUC) for the laser injury graph in figure 3A. Graph depicts mean with SEM. Analysis by one way ANOVA comparing to the no poloxamer control. \* $P \leq 0.05$  by ANOVA, \*\* $P \leq 0.003$  by ANOVA, \*\*\*\* $P \leq 0.0001$  by ANOVA,  $n=7-48$ . **C.** Representative images of muscle fibers before and 45 seconds after injury with multiphoton laser.



**Figure 4. A.** Results of laser injury assay performed in extensor digitorum longus (EDL) muscles of *mdx* mice, when exposed to poloxamers with more than 50% POE by weight. All curves represent fluorescence after injury while muscle was in 1X Tyrode's solution with 2mM  $Ca^{2+}$  and 100 $\mu$ M poloxamer. Graph depicts change in fluorescence over time of a lipophilic dye, FM4-64, with SEM. **B.** Area Under the Curve (AUC) for the laser injury graph in figure 4A. Graph depicts mean with SEM. Analysis by one way ANOVA comparing to the no poloxamer control. \*\*\*\* $P \leq 0.0001$  by ANOVA, # $P > 0.05$  by ANOVA,  $n=7-48$  **C.** Representative images of muscle fibers before and 45 seconds after injury with multiphoton laser.



**Figure 5. A.** Results of laser injury assay performed in human embryonic kidney (HEK) cells, when exposed to poloxamers P188 and P108. All curves represent fluorescence after injury while cells were in 1X Tyrode's solution with 2mM Ca<sup>2+</sup> and 100μM poloxamer. Graph depicts change in fluorescence over time of a lipophilic dye, FM4-64, with SEM. **B.** Area Under the Curve (AUC) for the laser injury graph in figure 5A. Graph depicts mean with SEM. Analysis by one way ANOVA comparing to the no poloxamer control. \*\*\*\*P ≤ 0.0001 by ANOVA, #P > 0.05 by ANOVA, n=6-11 **C.** Representative images of cells before and 45 seconds after injury with multiphoton laser.

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